Characterization of bacterial spore germination using phase-contrast and fluorescence microscopy, Raman spectroscopy and optical tweezers

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Published online 21 April 2011; doi:10.1038/nprot.2011.307

This protocol describes a method combining phase-contrast and fluorescence microscopy, Raman spectroscopy and optical tweezers to characterize the germination of single bacterial spores. The characterization consists of the following steps: (i) loading heat-activated dormant spores into a temperature-controlled microscope sample holder containing a germinant solution plus a nucleic acid stain; (ii) capturing a single spore with optical tweezers; (iii) simultaneously measuring phase-contrast images, Raman spectra and fluorescence images of the optically captured spore at 2- to 10-s intervals; and (iv) analyzing the acquired data for the loss of spore refractility, changes in spore-specific molecules (in particular, dipicolinic acid) and uptake of the nucleic acid stain. This information leads to precise correlations between various germination events, and takes 1–2 h to complete. The method can also be adapted to use multi-trap Raman spectroscopy or phase-contrast microscopy of spores adhered on a cover slip to simultaneously obtain germination parameters for multiple individual spores.

INTRODUCTION

Combination of optical tweezers and Raman spectroscopy, together with phase-contrast and fluorescence microscopy, could be very useful to noninvasively analyze and monitor physiological dynamics of single living cells (including microbial and mammalian cells) in an aqueous environment. Bacterial spores of *Bacillus* species, including those that cause food spoilage and food-borne disease[1], are formed in the process of sporulation[2]. These spores are dormant and very resistant to a variety of environmental stresses[3,4]. As a consequence, even in the absence of nutrients, these spores can survive for many years. However, when specific germinants are added, spores can rapidly return to life through the process of germination[5,6]. Germinated spores have lost the extreme resistance of dormant spores and are thus relatively easy to kill. A number of small molecules are accumulated to high levels in the central core of dormant spores, including the large depot of pyridine-2, 6-dicarboxylic acid (dipicolinic acid (DPA)) and its chelated divalent metal ions (predominantly Ca^{2+} (CaDPA)), that composes ~25% of the dry weight of the central core of the spores[7,8]. During spore germination, all CaDPA is released and then replaced in the core by water[5,6], and this makes up stage I of germination. CaDPA release then triggers stage II of germination, which is primarily the hydrolysis of the peptidoglycan cortex of the spore and full core rehydration[5]. After core rehydration is completed, metabolism and macromolecular synthesis begin in spore outgrowth[5,6]. Characterization of the details of the germination of individual spores could lead to better understanding of the mechanisms of spore germination and provide new ways to block germination and/or better ways to kill spores, thus preventing food spoilage and disease.

Several methods have been developed to examine the process of spore germination. The most common one is the measurement of the optical density at 600 nm of spore cultures, which falls by ~60% during germination[5]. Spectroscopic methods such as UV absorbance spectroscopy[9], Fourier-transform infrared spectroscopy[10] or fluorescence spectroscopy[11] (of DPA complexed with Tb^{+3}) have been used to measure DPA release from germinating spore populations. Atomic force microcopy has also been used to monitor changes in the spore surface during germination[12,13]. Other methods that are used to measure the germination of individual spores, including phase-contrast microscopy, fluorescence microscopy using a nucleic stain and Raman spectroscopy, are introduced in the following sections.

Phase-contrast microscopy

Contrast-enhanced images can be obtained by converting small variations in the optical phase of illumination light into corresponding changes in amplitude due to differences in refractive index between specimens and their environment[14]. For dormant spores, the water content in the core is very low (25–50% of wet weight, depending on the species)[7]. The high solid (small molecules)/water ratio in the spore core gives it a much higher refractive index compared with that of the environment (e.g., water). During spore germination, small molecule release and cortex hydrolysis allow core water uptake, resulting in a large decrease in the core's refractive index. As a consequence, dormant spores (high core refractive index) appear phase bright and germinated spores (low refractive index) appear phase dark. Consequently, phase-contrast microscopy has been widely used to monitor spore germination[15]. However, phase-contrast microscopy only reports physical changes in spore germination, and does not provide direct information on molecular changes.

Fluorescence microscopy

A high fluorescence intensity is obtained when a number of membrane-permeant stains bind to spore nucleic acids, in particular to DNA[16–18]. The SYTO 16 molecule, in particular, is a very effective nucleic acid stain that can readily distinguish dormant and germinated spores[19], as SYTO 16 cannot penetrate into the core of dormant spores, the site of spore nucleic acids. However, SYTO 16 uptake begins during germination and its binding to nucleic acids, predominantly DNA, in the core gives a strong green fluorescence on excitation at 460 nm. In previous work, measurement of the fluorescence of germinated spores due to nucleic acid stain

binding has been used to measure the kinetics of germination of spore populations[19–21]. However, again this method is unable to link nucleic acid stain binding to other germination events such as CaDPA release and cortex hydrolysis.

Laser tweezers Raman spectroscopy

Confocal or surface-enhanced Raman spectroscopy is a powerful method to analyze the molecular composition of various materials. Indeed, because of its high sensitivity, surface-enhanced Raman spectroscopy has been applied to the detection of CaDPA release from single germinating spores[22,23]. Optical tweezers allow the capture and manipulation of single biological particles by using the gradient force arising from a focused laser beam[24,25] and has been widely used for studies of cell sorting[26], cell motility[27] and molecular motors[28]. Confocal Raman spectroscopy combined with optical tweezers, called laser tweezers Raman spectroscopy (LTRS)[29–31], also allows quantitative measurement of CaDPA and other molecular components inside single spores, and thus reagentless identification of single spores in aqueous solution, as well as monitoring the kinetics of events during germination of single trapped spores[32–34].

Combinations of different methods to follow spore germination

A few methods have also been developed to provide multimodal information for better understanding of the germination of bacterial spores. Confocal scanning laser microscopy has been used to measure both the loss of refractility and changes in permeability to ethidium bromide of individual *Bacillus cereus* spores during germination[35]. The combination of LTRS and elastic light scattering has also been used to link CaDPA release to changes in spore morphology and refractive index during germination[33]. Recently, a combination of phase-contrast microscopy, optical tweezers and Raman spectroscopy has been developed[34]. With this technique, changes in refractility of individual spores during germination were assessed with phase-contrast imaging, while Raman spectroscopy gave information on changes in spore-specific molecules (e.g., CaDPA release). This method found that the end of the rapid drop in spore refractility during germination precisely corresponds to the completion of CaDPA release. In addition, the temporal resolution of the LTRS system was improved to ~2 s, allowing a very precise delineation of dynamic changes during the germination process. An extension of this technique combining quantitative differential interference contrast microscopy with Raman spectroscopy has also been developed[36]. A more recent refinement of the LTRS technique is to combine this with fluorescence microscopy[37], allowing analysis of stain uptake into the spore core and its binding to nucleic acids during germination.

In this protocol, we present a methodology that combines phase-contrast microscopy, fluorescence microscopy, Raman spectroscopy and optical tweezers to monitor the germination of single *B. cereus* spores. Multi-trap LTRS array[38] will also be described to characterize the germination of multiple individual spores via real-time Raman spectra, refractility, and fluorescence images, as well as use of spore refractility to simultaneously monitor the germination of hundreds of individual spores. As mentioned above, phase-contrast microscopy measures changes in spore refractility due to CaDPA release and cortex hydrolysis, Raman spectroscopy measures changes in molecular composition (e.g., CaDPA release) and fluorescence microscopy monitors the uptake of a fluorescent dye and its binding to spore nucleic acids. Combination of these three methods will precisely correlate the kinetics of CaDPA release, cortex hydrolysis and nucleic acid stain uptake and binding during spore germination, providing a better understanding of the overall germination process. The use of optical tweezers with low-power near-infrared lasers allows the holding of single spores in an aqueous germination solution for hour-long measurements with phase-contrast and fluorescence microscopy, as well as with Raman spectroscopy.

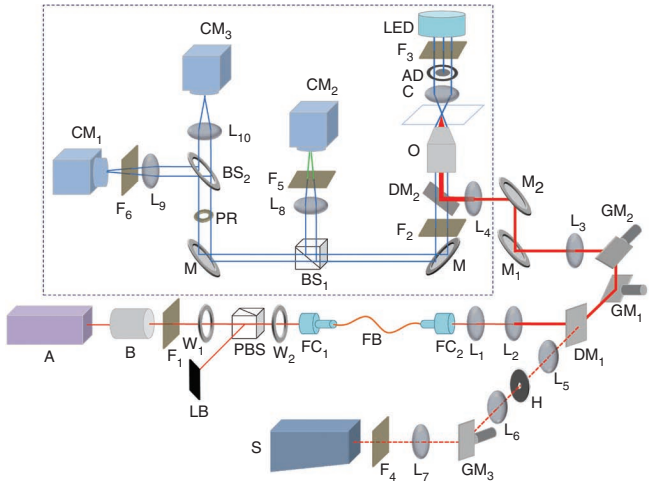

Figure 1 | Scheme of the experimental setup. See **Table 1** for abbreviations. The components outlined by the dashed line are the microscope optics. The solid red line, dotted red line, solid blue line and solid green line indicate, respectively, the Raman excitation and optical tweezers laser beam, the backward Raman scattering light, the phase-contrast imaging and fluorescence excitation light, and the fluorescence light.

The protocol details the following steps for characterizing spore germination: loading heat-activated spores and germinant solution; capturing single or multiple individual spores; acquiring time-lapse phase-contrast images, fluorescence images and Raman spectra of the individual spores; and quantifying the loss of spore refractility, uptake of fluorescent nucleic acid stains, and release of CaDPA. We also provide detailed procedures for constructing the confocal LTRS system using phase-contrast and fluorescence microscopes, monitoring the dynamic germination processes of single spores in aqueous germinants using LTRS and phase-contrast/fluorescence imaging and simultaneously characterizing the germination of hundreds of individual spores using phase-contrast imaging. A major advantage of the methodology presented here is the simultaneous measurement of multimodal information for single cell dynamics in general. Apart from bacterial spore germination, the combined setup described here could also be used to obtain real-time information on molecular composition, cell refractility, levels of DNA and its accessibility, as well as dynamic movement of fluorescently tagged proteins in other single microbial cells suspended in liquid, including changes during the cell cycle of synchronized cells or during other microbial differentiation processes, for example, that of swarmer cell formation in *Caulobacter crescentus*[39].

Experimental design

Combination of laser tweezers and Raman spectroscopy. The LTRS system is able to measure the Raman spectra of single or multiple individual spores in aqueous solution using single or multiple traps. The design of the LTRS system is shown in **Figure 1** (see **Table 1** for abbreviations used throughout). The single laser beam at 780 nm from a diode laser (A) is used for both Raman

TABLE 1 | Abbreviations of equipment.

Abbreviations	Equipment or element description
A	Diode laser at 780 nm
B	Optical isolator
F_1	Laser line filter at 780 nm
W_1, W_2	Half-wave plate mounted on rotation mounts
PBS	Polarizing cube beam splitter
LB	Laser beam block
FC_1, FC_2	Fiber coupler/collimator
FB	Single-mode optical fiber
L_1	The first lens for laser beam expansion (f = −25 mm)
L_2	The second lens for laser beam expansion (f = 75 mm)
L_3	Scan lens (f = 100 mm)
L_4	Tube lens (f = 200 mm)
L_5	Lens before pinhole (H) (f = 100 mm)
L_6	Lens after pinhole (H) (f = 75 mm)
DM_1	Dichroic mirror
DM_2	Dichroic hot mirror (>95% reflection for 700–1,150 nm)
GM_1, GM_2	XY galvanometer mirrors
M_1, M_2	Broadband dielectric mirrors (750–1,100 nm)
H	Pin hole (100 μm), mounted on a translation stage and an *XY* translator with micrometer drive
GM_3	Galvanometer mirror (1D)
M	Mirrors inside the Nikon microscope (reflection for visible light)
F_2	Band-pass filter
O	Objective (×60, NA = 1.4)
C	Condenser for the Nikon microscope
AD	Annular diaphragm
F_3, F_6	Excitation filter (λ = 455 nm, 10 nm bandwidth)
LED	Collimated light-emitting diode for fluorescence excitation (455 nm, 140 mW)
F_4	Long-pass Raman filter
L_7	Lens for Raman spectroscopy measurements (f = 75 mm), mounted on a translation stage and an *XY* translator with micrometer drive
S	Imaging spectrograph, equipped with back-illuminated deep-depletion CCD camera
BS_1	Beam splitter prism inside the Nikon microscope (80% reflection and 20% transmission)
F_5	Emission filter
PR	External phase contrast ring
BS_2	Plate beam splitter (50%/50% reflection/transmission)
L_8 – L_{10}	Imaging lens inside the Nikon microscope
CM_1	TEC (thermoelectric cooling) cooled CCD camera for phase-contrast images
CM_2	TEC (phase-contrast) cooled CCD camera for fluorescence images
CM_3	Video CCD camera for TV monitor

spectroscopy excitation and the optical tweezers. An optical isolator (B) is used to block the feedback light, a narrow band-pass filter (F_1) is used to spectrally purify the excitation laser beam and a pair of half-wave plates (W_1, W_2) and a polarizing beam splitter (PBS) are used to adjust the intensity and polarization direction of the laser beam. To obtain good spatial quality, a single-mode polarization-maintaining fiber (FB) together with fiber connectors (FC_1, FC_2) is used to deliver the laser beam. The laser beam diameter from FB is expanded with a pair of lenses (L_1, L_2), reflected by a dichroic mirror (DM_1) and a pair of galvanometer mirrors (GM_1 and GM_2), and passes through a pair of lenses (L_3, L_4). The laser beam is then introduced into the objective (O; ×60, NA = 1.40) of an inverted microscope (Nikon TiS) through a hot mirror (DM_2) to form single-trap optical tweezers. A spore is trapped in the focus of the focused laser beam and Raman scattering light is excited with the same laser beam. The trapping of a single spore can be easily carried out by moving the microscope stage such that a spore in the aqueous sample is moved to be near the focus of the laser beam and then captured by the gradient force. A video charge-coupled device (CCD) camera (CM_3) is used to guide the movement of the stage and verify the trapping of a single spore in the laser focus (see Step 18). The backward Raman scattering light from the trapped spore that has wavelengths longer than 800 nm is collected with the same objective (O) and separated from the 780 nm excitation laser with the dichroic mirror (DM_1), and then spatially filtered with a 100-μm pinhole (H) and a pair of lenses (L_5, L_6) to eliminate the out-of-focus light from the specimens. The Raman scattering beam is then reflected by another galvanometer mirror (GM_3), spectrally filtered by a long-pass filter (F_4) and focused onto the entrance slit of an imaging spectrograph (S), which contains a CCD detector (PIXIS 400BR, Princeton Instruments) to record the Raman spectra of the trapped spore. Typical results using the LTRS system to trap a single spore and record its Raman spectrum are shown in **Figure 2**.

An LTRS array can be generated by rapidly scanning the single laser beam with two galvanometer mirrors (GM_1 and GM_2) in *x* and

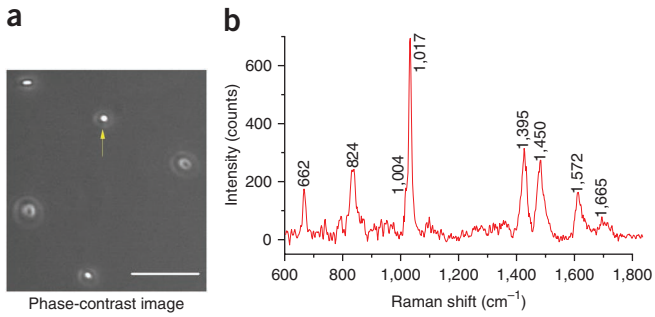

Figure 2 | Phase-contrast image and Raman spectrum of an individual trapped dormant *B. cereus* spore. (**a**) Phase-contrast images of dormant *B. cereus* spores suspended in solution (scale bar, 10 μm). The spore indicated by the arrow is the one being optically trapped. The trapped spore looks circular as it is aligned along the lateral direction and randomly rotates in the optical trap. The trapped spore is on the imaging focus of the CCD camera; the other spores are floating in the solution with different alignments, and some are out of the imaging focus. (**b**) Raman spectrum of a single trapped dormant *B. cereus* spore. The background spectrum without the spore in the trap has been subtracted. The unit of the intensity is the photon counts of the spectrograph with 3-mW laser power (measured at the position between the *XY* galvanometer mirrors (GM_1, GM_2) and the scan lens, L_3) and 2-s exposure time.

y directions, with a programmable pattern to create time-averaged multiple optical traps, while another galvanometer mirror (GM_3) is synchronously steered to project the backward Raman scattering from each trapped spore to different positions on the entrance slit of the spectrograph (S) such that Raman spectra of these trapped spores are simultaneously acquired by a CCD detector[38]. Either a 1D or 2D LTRS array can be generated. For example, by scanning the GM_1 with a two-step staircase waveform and scanning the GM_2 with a four-step staircase waveform, a 2 × 4 array of traps is created, as shown in **Figure 3a**. The corresponding Raman spectra of these eight trapped *B. cereus* spores are projected on eight different vertical positions of the spectrograph's CCD chip (**Fig. 3b**) by synchronously steering the GM_3 with an eight-step staircase waveform. The multi-trap design greatly improves the measurement efficiency of the LTRS system. To avoid the complex description of this protocol, we will primarily describe procedures for application of the single-trap LTRS, and the multi-trap LTRS can easily be adapted from this (see ref. 38 for details).

Combination of phase-contrast and fluorescence microscopy with LTRS. The optical design for phase-contrast and fluorescence microscopy is outlined inside the dashed line in **Figure 1**. It is not straightforward to combine phase-contrast microscopy with Raman spectroscopy and fluorescence microscopy, as a phase plate is usually placed on the back pupil of phase objectives and this blocks much of the extremely weak Raman scattering light and weak fluorescence light. The phase objective also blocks the incident laser beam and degrades the performance of optical tweezers. Here, we use an inverted microscope with the external phase-contrast design, in which the phase ring (PR) is placed in an intermediate image plane of the microscope. By using this external phase configuration, a normal high-throughput objective without a phase ring can be used to collect the Raman scattering light and the fluorescence light, and to form the optical tweezers[34]. A collimated light emitting diode (LED) at 455 nm is used to replace the illumination lamp of the microscope, serving as both the illumination light for phase-contrast microscopy and the excitation light for fluorescence microscopy[37]. After passing through the excitation filter (F_3; to remove the LED background at ~530 nm, which would clash with the emission band of the green fluorescent nucleic acid-SYTO 16 complex) and the annulus diaphragm (AD), the LED beam is focused on the specimen by a condenser (C) with an intensity of ~4.0 mW cm^{-2}. A custom-mounted dichroic mirror (DM_2) below the objective is used to separate the overlapped phase-contrast imaging light at 455 nm and the fluorescent light at 530 nm from the elastic scattering light at 780 nm and the backward Raman scattering light above 800 nm. A band-pass filter (F_2) is used to block the residual elastic scattering light in the imaging optical path. Of the overlapped imaging/fluorescent light, 80% is reflected to the left side port of the microscope by a beam-splitter prism (BS_1) and filtered with an emission filter (F_5) to remove the illumination light at 455 nm such that only the green fluorescent light is focused by a lens (L_8) onto and detected by a CCD camera (CM_2) to acquire the fluorescence images. The transmitted portion of the illuminating light passes through BS_1 and an external phase ring plate (PR), and then is divided into two beams by a 50% beam-splitter mirror (BS_2). The reflected light is spectrally filtered with a narrow-band interference filter (F_6) at 455 nm to remove the green fluorescence light (at 530 nm), and is then focused by a lens (L_9) onto a CCD camera (CM_1) to acquire the phase-contrast images on the side port. The transmitted imaging light is focused by a lens (L_{10}) onto a video camera (CM_3) on the top port for monitoring, and this camera replaces the eyepieces.

Monitoring the germination of single optically trapped spores. The *B. cereus* spore sample is first heat-activated at 65 °C for

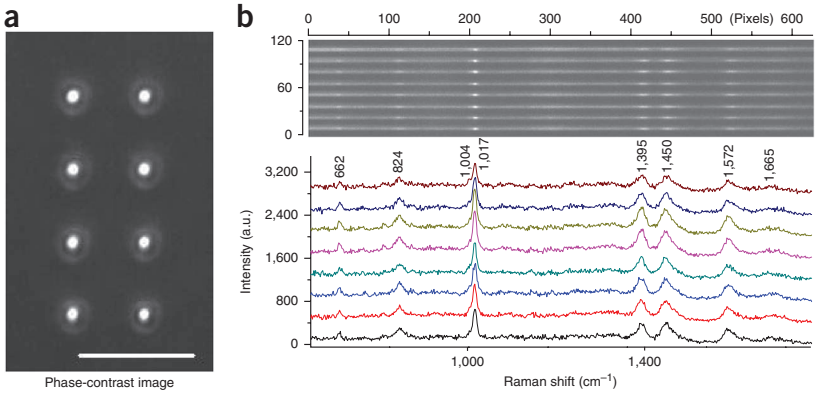

Figure 3 | Phase-contrast and Raman spectral images of multiple trapped dormant *B. cereus* spores. (**a**) Phase-contrast image of eight trapped dormant *B. cereus* spores in a 2 × 4 optical tweezers array (scale bar, 10 μm). The laser beam scans across these trap positions with a frequency of 100 Hz and a total laser power of 16 mW. (**b**) Top: Spectral image of Raman scattering light from the eight trapped dormant *B. cereus* spores in **a** recorded simultaneously by the spectrograph CCD camera at one exposure (exposure time of 3 s). Bottom: The corresponding Raman spectra of individual spores in the top spectral image obtained by binning the vertical pixels (9 pixels) of each spectral image.

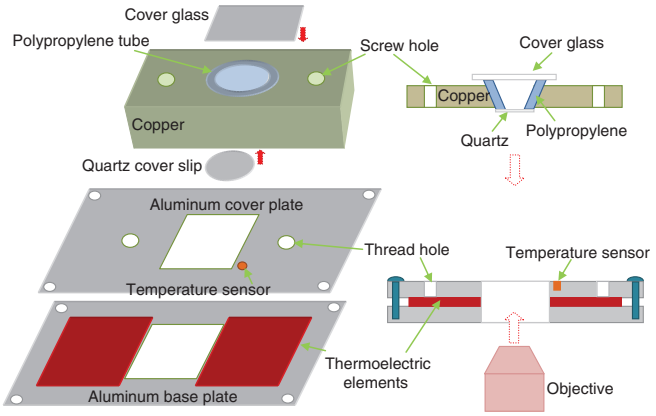

Figure 4 | Sample container and heating stage designed for temperature control. Left, an exploded view; right, a section plane view.

30 min and then placed at 0 °C for 15 min before measurements. The heat-activated dormant spores are loaded into a temperature-controlled microscope sample holder that contains a germinant solution and SYTO 16 nucleic acid stain (**Fig. 4**). A single spore is then captured in the optical trap and held for 1–2 h until it germinates (see PROCEDURE Step 18 for verifying the capture of a spore). The time-lapse Raman spectra and the fluorescence images are recorded synchronously at a rate of one frame per 7 s, and the phase-contrast images are recorded at a rate of 1 frame per 5 s (**Fig. 5**). The exposure time periods for Raman, fluorescence and phase-contrast image acquisitions are set as 5, 3 and 0.2 s, respectively. Following each exposure, a small portion of the phase-contrast image (21 × 21 pixels; **Fig. 5b**) and the fluorescence image (25 × 25 pixels; **Fig. 5c**), both corresponding to an area of 2.5 μm × 2.5 μm in which the trapped spore is located, are selected. The intensities of the phase-contrast and fluorescence images within the selected area are first summed and averaged over the selected pixels and the averaged background values of nearby pixels are subtracted. These calculated intensities are then displayed to monitor germination in real-time. The CaDPA level of the trapped spores is calculated from the peak intensity of the CaDPA-dominated band at 1,017 cm^{-1} in Raman spectra, after subtraction of the smoothed background level at 990 cm^{-1} (**Fig. 5a**). The above procedures are repeated for monitoring other individual spores in additional experimental runs.

Simultaneously determining the time, $T_{release}$, for completion of CaDPA release from large numbers of spores germinating on cover slips. The germination of large numbers of individual spores can also be analyzed simultaneously using phase-contrast microscopy. A drop of a heat-activated spore suspension is placed on the

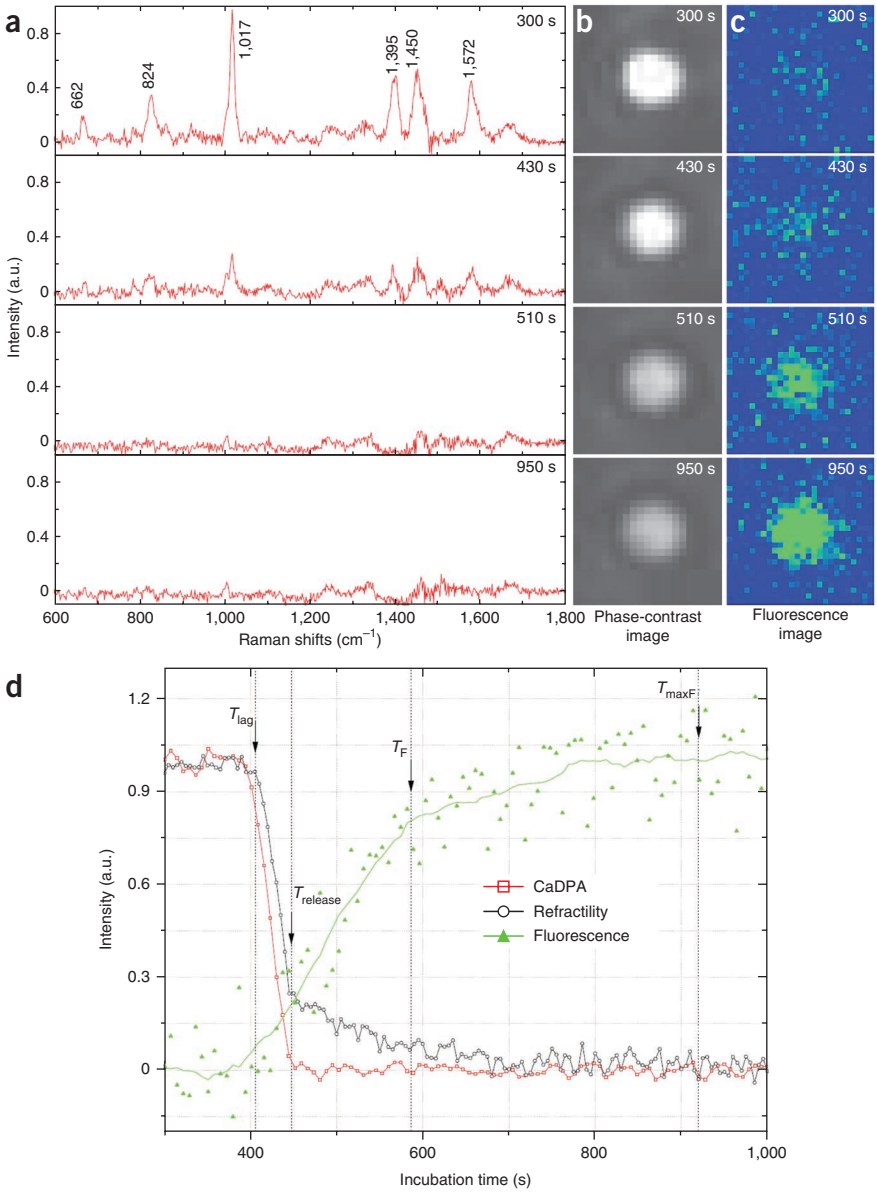

Figure 5 | Simultaneous recording of Raman spectra, phase-contrast images and fluorescence images of a single optically trapped *B. cereus* spore germinating at 24 °C with 1 mM L-alanine plus 0.5 μM SYTO 16 in 25 mM Tris-HCl buffer (pH 7.4). (**a**) Sequential Raman spectra. The indicated peaks at 662, 824, 1,017, 1,395 and 1,572 cm^{-1} are bands that are almost exclusively because of CaDPA. (**b**) Sequential phase-contrast images, 21 × 21 pixels (2.5 × 2.5 μm^2). (**c**) Sequential fluorescence images, 25 × 25 pixels (2.5 × 2.5 μm^2). (**d**) Intensities of CaDPA, refractility and fluorescence as a function of germination time. The CaDPA level is calculated from the intensity of the Raman band at 1,017 cm^{-1} and normalized to the value at the first time of measurement. Refractility is calculated by normalizing the spore's phase-contrast image intensity to its value at the first time of measurement (corresponding to that of the dormant spore) after subtraction of the last unchanged image intensity value (corresponding to that of the fully germinated spore)[34]. Fluorescence intensity is normalized to the maximum intensity value. The time periods indicated by the arrows are defined as: T_{lag}, the time of initiation of rapid CaDPA release; $T_{release}$, the time for completion of rapid CaDPA release; T_F, the time at the end of the rapid increase in SYTO 16 fluorescence; and T_{maxF}, the time at which the SYTO 16 fluorescence reached its maximum value[37].

BOX 1 | CUSTOM-MADE PROGRAMS FOR THE EXPERIMENTAL CONTROL AND DATA ANALYSIS

1. A program written in Visual Basic 6.0 is used to control the charge-coupled device camera (CM_1) for capturing the phase-contrast images, display the images, store the images both in JPEG file format and as text files (16-bit), calculate and then display the intensity values of the phase-contrast images of a trapped spore from the text image for the real-time monitoring of spore's germination process.
2. A program written in MATLAB (R2008a) is used to control the multifunction data acquisition board to generate the analog trigger signals for the synchronous recording of the fluorescence images and the Raman spectra, and the synchronous analog voltage signals to drive the galvanometer mirrors. This program also calculates and displays the intensity values of the fluorescence images of a trapped spore for the real-time monitoring of spore's fluorescence change.
3. One data processing program written in MATLAB (R2008a) is used to analyze the Raman spectra to calculate the CaDPA level through the CaDPA-dominated Raman band at 1,017 cm^{-1}. The other data processing program written in MATLAB (R2008a) is used to analyze the phase-contrast images of large amount of bacterial spores adhered on the cover slip to get the refractility change of each individual spore during the germination. (See **Supplementary Methods**. This file contains the code for the three programs.)

surface of a glass cover slip (glued on a sample container) and air-dried so that the spores adhere firmly on the cover slip. The germinant solution (~300 µl; 10 or 0.1 mM L-alanine in 25 mM Tris-HCl buffer (pH 7.4)) is added above the cover slip–adhered spores in the sample container, which is placed on the heating stage mounted on the microscope stage and incubated at 24 °C. The full-view field (1,600 × 1,200 pixels; an area of 192 × 144 μm^2) of the phase-contrast images of large numbers of spores germinating on the cover slips are recorded at a rate of 1 frame per 15 s, with an exposure time of 0.2 s. Several runs of replicate experiments are conducted to accumulate data for ≥ 500 individual spores. Recorded images are analyzed by the MATLAB program (See **Box 1** in EQUIPMENT SETUP and the **Supplementary Methods** file) to locate and label individual spores (**Fig. 6a**,**b**), and to calculate phase-contrast intensities of each individual spores. The values for $T_{release}$ (**Fig. 6c**) for the individual spores are extracted using Origin software to plot the changes in intensity of phase-contrast images for each spores.

In the PROCEDURE, Steps 1–29 describe the construction of the LTRS setup combined with external phase-contrast and fluorescence microscopy. Steps 30–51 describe the characterization of single spore germination with the constructed setup. For the characterization of single spore germination with phase-contrast microscopy only (without the use of LTRS and fluorescence microscopy), start from Step 52.

MATERIALS

REAGENTS

- *B. cereus* T was originally obtained from H.O. Halvorson, and spores were prepared at 30 °C in a defined liquid medium and purified such that the purified spores were free (≥ 98%) of germinated spores, growing or sporulating cells and cell debris[40,41].
- Distilled water and deionized water
- L-alanine (99%, Acros Organics, Fisher Scientific, cat. no. AC10283-0250)
- SYTO 16 green fluorescent nucleic acid stain (Invitrogen, cat. no. S-7578)
- Tris-HCl buffer (1 M, pH 7.4, Teknova, Fisher Scientific, cat. no. 50843329)
- Optical adhesive (NOA68, Norland Product)
- Ethanol
- Bleach

EQUIPMENT

- To construct the LTRS system combined with phase-contrast and fluorescence microscopy, we use the following equipment, as well as a high-power external cavity Diode laser (780 nm, 500 mW, Sacher Lasertecknik, model no. TEC-300-0780-0500).
- Optical table (size 1,200 mm × 1,800 mm × 203 mm, Newport, model no. M-RS2000-46-8). A lab space (~300 ft^2) that can contain the optical table and three desktop computers is suitable for the whole experiment.
- Inverted microscope with external phase-contrast unit (Nikon TiS, Nikon, model no. Eclipse TiS) ▲ **CRITICAL** An external phase-contrast design for the microscope is essential.
- High-throughput imaging spectrograph (with a 1,200 g mm^{-1} grating; Princeton Instruments, cat. no. LS-785)
- Back-illuminated deep depletion CCD camera (Princeton Instruments, cat. no. PIXIS 400BR), equipped with spectrum acquisition software (WinSpec/32)
- Thermoelectric cooling (TEC) cooled CCD camera for phase-contrast images (QSI, model no. 520), equipped with image acquisition software (custom-made program in Visual Basic 6.0, see **Box 1** in EQUIPMENT SETUP and the **Supplementary Methods** file.)
- TEC cooled CCD camera for fluorescence images (Opticstar, model no. DS-145M ICE), equipped with image acquisition software (Nebulosity)
- Video CCD camera for TV monitor (Panasonic, model no. GP-KR222)
- Thermistor (10 KΩ; Thorlabs, cat. no. TH10K)
- Digital-controlled water bath (Fisher Scientific, cat. no. 15-462-5Q)
- Collimated diode laser at 850 nm for Raman optical path alignment (Thorlabs, cat. no. L850P010)
- Multifunction data acquisition board (Measurement Computing, model no. USB-1208HS-4AO)
- Three computers with the Windows XP operating system for experimental control and data acquisition. One computer equipped with spectrum acquisition software (WinSpec/32) controls the Raman spectrograph, the second computer equipped with the Visual Basic 6.0 program controls the phase contrast image CCD camera and the third computer equipped with the MATLAB program controls the fluorescence image CCD camera and the multifunction data acquisition board. (See **Box 1** in EQUIPMENT SETUP and the **Supplementary Methods** file, which contains the code for each custom-made program.)
- Glass cover slips (size 22 mm × 22 mm, thickness 0.08–0.13 mm, Fisher Scientific, cat. no. NC9115219)
- Quartz cover slips (diameter 10 mm × 0.1 mm, UQG (Optics), cat. no. CFQ-1010)
- Thermoelectric elements (Thorlabs, cat. no. TEC3-6)
- Heat sink grease (RadioShack, cat. no. 276-1372)
- Temperature sensor (Thorlabs, cat. no. AD590)
- Temperature controller (Thorlabs, model no. TEC2000)
- Optical isolator (ISOWAVE, model no. 1-80-14-L)
- Laser line filter (780 nm; Semrock, cat. no. LL01-780-12.5)

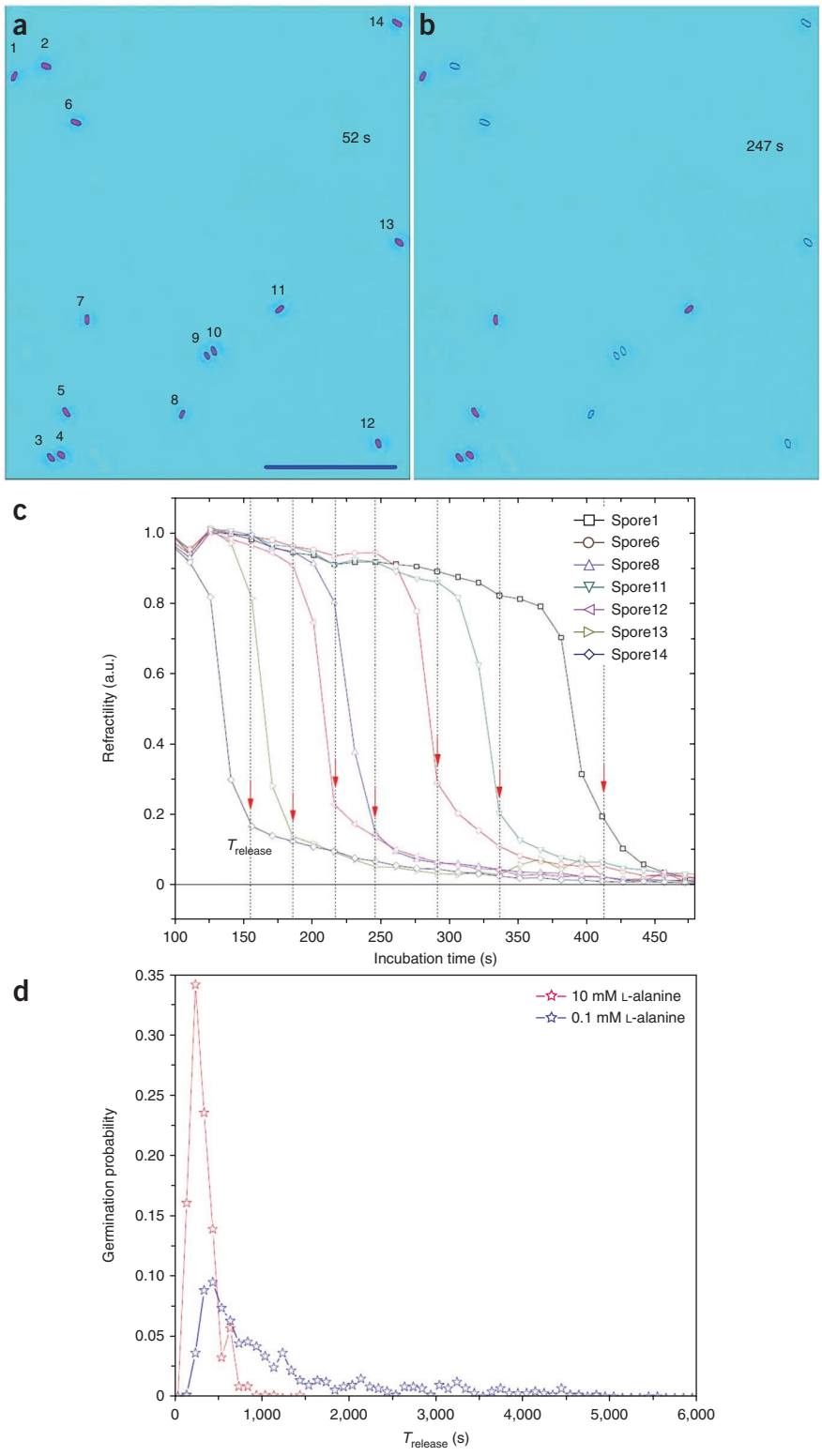

Figure 6 | Germination heterogeneity characterized by time of $T_{release}$. $T_{release}$ times were obtained simultaneously from phase-contrast images of large numbers of *B. cereus* spores adhered on a glass cover slip and germinated with L-alanine in Tris-HCl buffer at 24 °C. (**a**) A selected phase-contrast image area of the cover slip with adhered *B. cereus* spores, 52 s after starting germination with 10 mM L-alanine (scale bar, 20 µm). The phase-bright images (pseudocolored purple) of individual spores are outlined by blue circles using the MATLAB program, with which the phase-contrast intensities of individual spores are calculated. (**b**) The same phase-contrast image area in **a**, 247 s after addition of 10 mM L-alanine. The blue circles are the same as in **a**; note that a number of the spores are no longer phase bright, as they have germinated. (**c**) Loss of refractility of multiple individual spores in **a** and **b** during germination with 10 mM L-alanine. Red vertical arrows indicate the times of $T_{release}$. Refractility is calculated by normalizing the phase-contrast image intensity of the spore to its value at the first time of measurement (corresponding to that of the dormant spore) after subtraction of the last unchanged image intensity value (corresponding to that of the fully germinated spore). (**d**) Probability distributions of $T_{release}$ values for large numbers of individual *B. cereus* spores germinating with either 10 mM L-alanine (red, 500 spores) or 0.1 mM L-alanine (blue, 900 spores).

- Half-wave plate (Casix, cat. no. WPZ1210-L/2-780+RMW0210) mounted on rotation mounts (Thorlabs, cat. no. RSP1C)
- Polarizing cube beam splitter (Edmund Optics, cat. no. NT49-872)
- Fiber coupler/collimator (Thorlabs, cat. no. CFC-8X-B)
- Single-mode optical fiber (Thorlabs, cat. no. P3-780PM-FC-2)
- First lens for laser beam expansion ($f = -25$ mm; Thorlabs, cat. no. LD2297-B)
- Second lens for laser beam expansion ($f = 75$ mm; Thorlabs, cat. no. LB1901-B)
- Scan lens ($f = 100$ mm; Thorlabs, cat. no. AC254-100-B)
- Tube lens ($f = 200$ mm; Thorlabs, cat. no. AC254-200-B)
- Lens before pinhole ($f = 100$ mm; Thorlabs, cat. no. AC254-100-B)
- Lens after pinhole ($f = 75$ mm; Thorlabs, cat. no. AC254-075-B)
- Dichroic mirror (Semrock, cat. no. LPD01-785RU-25)
- Dichroic hot mirror (>95% reflection for 700–1,150 nm; Edmund Optics, cat. no. NT64-467)
- *XY* galvanometer mirrors (Cambridge Technology, model no. 6220H)
- Broadband dielectric mirrors (750–1,100 nm, Thorlabs, cat. no. BB1-E03)
- Pinhole (100 µm, Edmund Optics, cat. no. NT56-283), mounted on a translation stage (Thorlabs, cat. no. PT1A) and an *XY* translator with micrometer drive (Thorlabs, cat. no. ST1XY-S)
- Galvanometer mirror (1D; Thorlabs, model no. GVS001)
- Band-pass filter (Edmund Optics, cat. no. N46-433)
- Objective (×60, NA = 1.4; Plan Apo, Nikon)
- Annular diaphragm (Nikon, code Ph3)
- Excitation filter (λ = 455 nm, 10 nm bandwidth; Edmund Optics, cat. no. NT62-143)
- Collimated light-emitting diode for fluorescence excitation (455 nm, 140 mW; Thorlabs, model no. LEDC3)
- Long-pass Raman filter (Semrock, cat. no. LP02-780RU-25)
- Lens for Raman spectroscopy measurements ($f = 75$ mm; Thorlabs, cat. no. AC254-075-B), mounted on a translation stage (Thorlabs, cat. no. PT1A) and an *XY* translator with micrometer drive (Thorlabs, cat. no. ST1XY-S)

- Emission filter (Chroma, cat. no. HQ530/30m)
- External phase-contrast ring (Nikon, ×60/Ph3)
- Plate beam splitter (50%/50% reflection/transmission; Edmund Optics, cat. no. NT43-736)
- MATLAB software
- Origin software

REAGENT SETUP

Spore preparation A culture of *B. cereus* in CCY broth (250 ml in a 2-liter flask, inoculated with 1 ml of a mid-log phase Luria broth culture grown at 30 °C) was incubated with continuous shaking at 30 °C for 2 d until > 90% free spores were present[40,41]. Spores were harvested and washed multiple times by centrifugation and resuspension in 4 °C distilled water over a period of a week, discarding the upper layer of cellular debris in the pellet from early washing steps. The final purified spores were stored at 3–5 mg (dry weight) per ml in distilled water at 4 °C and protected from light[40,41]. These spores should be free (≥ 98%) from germinated spores, growing or sporulating cells and cell debris, as observed by microscopy, and contain 2–4 × 10^9 spores per ml.

Germinant solution Dissolve and stir 8.91 g of L-alanine in 100 ml of deionized water to prepare 1 M L-alanine. Dilute 1 M L-alanine, 1 M Tris-HCl buffer (pH 7.4) and SYTO 16 (1 mM solution in DMSO) with deionized water to form germination solutions of 10 mM, 1 mM or 0.1 mM L-alanine in 25 mM Tris-HCl buffer. For fluorescence experiments, the germination solution contains 0.5 μM SYTO 16. Mixed germination solution can be stored at 4 °C for 1 week, protected from light. **! CAUTION** SYTO 16 dye is a potential mutagenic agent; use latex gloves to handle DMSO stock.

Clean and sterilize the sample container with the glued quartz cover slip After each experiment, add household bleach (contains 5% (wt/vol) sodium hypochlorite, ~300 μl) to the sample container with the glued quartz cover slip, incubate for 5 min to sterilize it and then wash three times with distilled water. Use 99% (vol/vol) ethanol to wash and clean the sample container and the quartz cover slip three times, followed by three washes with distilled water. Use lens tissues with 99% (vol/vol) ethanol to clean the surface of the quartz cover slip three times, followed by three washes with distilled water. Air-dry the quartz cover slip and sample container in a cabinet for the next use.

EQUIPMENT SETUP

Sample container A polypropylene tube cut flat on both ends (bottom diameter 8 mm, top diameter 12 mm, height of 4 mm) is placed inside a copper plate (50 mm × 30 mm × 3.8 mm; see **Fig. 4**); a fused quartz cover slip (UQG, diameter 10 mm × 0.1 mm) is adhered to the bottom of the polypropylene tube with an adhesive (Norland) cured with an ultraviolet light lamp, and the top of the polypropylene tube is covered by a glass cover slip (22 mm × 22 mm × 0.15 mm). The resulting container can hold ~300 μl of germination solution. The copper plate is used to heat the germination solution by thermal contact with a heating stage. Two holes in the copper plate are used for two screws to affix the sample container on the heating stage. **▲ CRITICAL** Use a quartz cover slip to reduce the background Raman signal caused by the microscope cover slip. The sample container is designed to avoid the copper being in contact with the germination solution.

Heating stage Two thermoelectric elements (Thorlabs) are placed between two pieces of aluminum plate (150 mm × 100 mm × 2 mm), with heat sink grease (Radio Shack) applied on the interfaces between the thermoelectric modules and the aluminum plates (see **Fig. 4**). The whole stage is assembled by four screws. A temperature sensor (Thorlabs) is mounted on the cover aluminum plate to measure the temperature of the heating stage. The thermoelectric elements and the temperature sensor are controlled by a temperature controller (Thorlabs) to stabilize the temperature of the sample container from 20 to 80 °C, with a resolution of 0.2 °C.

Computer program See **Box 1** and the **Supplementary Methods** file.

PROCEDURE

Construct optical tweezers with the external phase-contrast microscope ● TIMING 16 h

1| *Modify and optimize the external phase-contrast microscope (Steps 1–6):* Install and fix the microscope on the optical table. Install the dichroic mirror (DM_2) with a 45° mirror mounted on the epifluorescence filter turret mount (under the nosepiece) of the microscope.

2| Install the tube lens (L_4, f = 200 mm) inside the epifluorescence illumination mount of the microscope base with a hollow cylinder mount. Set the distance between the objective (O) and the tube lens (L_4) at 200 mm.

3| Install the LED with an adaptor on the lamphouse of the microscope to replace the original halogen lamp. Install the mirror (M_2) with a 45° mirror mount on the rear of the microscope to reflect the laser beam into the microscope.

4| Power and turn on the LED. Install the objective (Plan Apo objective, ×60, NA = 1.4) in the optical path. Center the external phase-contrast ring (PR) and the condenser annular diaphragm (AD) according to the manufacturer's manual for phase-contrast microscopy.

5| Install the beam splitter (BS_2) and video camera (CM_3) on the top port of the eyepiece tube mount. Connect the video camera (CM_3) to a TV monitor. Adjust the video camera (CM_3) position to ensure that the focus on the video camera is the same as the focus through the eyepiece.

6| Verify the external phase-contrast imaging using a test glass cover slip glued on the sample container, i.e., spread 2 μm polystyrene spheres on the test glass cover slip or dry a drop of dormant *B. cereus* spores on the test glass cover slip and then add water on the top of the cover slip. The polystyrene spheres and dormant spores should appear bright in phase-contrast images.

7| *Align the laser beam in the microscope and optimize the single-beam optical tweezers (Steps 7–19):* Install the diode laser (A) on the optical table. Wear laser goggles for safety. Turn on the laser. Set the laser current slightly above the threshold current. Install the optical isolator (B) in front of the laser to block the back reflection into the laser. Install the laser line filter (F_1; at 780 nm) after the optical isolator to suppress ambient light.

! CAUTION It is a high-power near-infrared diode laser; comply with all relevant laser safety guidelines.

8| Install the half-wave plate (W_1) with a rotation mount and install the polarizing cube beam splitter (PBS). Rotate the half-wave plate (W_1) to adjust the laser power to 5 mW for the alignment.
! CAUTION A part of the laser beam comes out from the side of the polarizing cube beam splitter (PBS), which is out of the optical path. Make sure to block the laser beam with a beam block (LB) for safety.

9| Install 2 m of single-mode polarization-maintaining fiber (FB) to transport the laser for a better spatial mode. Use one adjustable aspheric lens collimator (FC_1) as the fiber coupler to couple the laser beam into the fiber and another one for the laser output collimator (FC_2). Use a pair of mirrors in front of the fiber to align the laser beam into the fiber coupler. Adjust the aspheric lens of the fiber coupler (FC_1) to optimize the laser coupling. Adjust the aspheric lens of the fiber output collimator (FC_2) to optimize the collimation of the output laser. Place the half-wave plate (W_2) on the rotation mount in front of the fiber coupler. Rotate the half-wave plate (W_2) to align the polarization direction of the laser beam (linear polarization) along the slow axis of the fiber.

10| Install the dichroic mirror (DM_1) at a 45° incident angle to the laser beam. It reflects the laser beam while allowing the transmission of the backward Raman scattering light. Insert two lenses (L_1, $f = -25$ mm and L_2, $f = 75$ mm) between the fiber output collimator (FC_2) and the dichroic mirror (DM_1) to expand the laser beam diameter from 1.4 mm to 4.2 mm.

11| Install the XY galvanometer mirrors (GM_1, GM_2). Install the mirror (M_1) with a 45° mirror mount. Insert the scan lens (L_3, $f = 100$ mm) between the XY galvanometer mirrors (GM_1, GM_2) and the mirror (M_1). Set the distance between the tube lens (L_4) and the scan lens (L_3) at 300 mm. Set the distance between the scan lens (L_4) and the XY galvanometer mirrors (GM_1, GM_2) at 100 mm. Power the galvanometer motors and apply 0 V control voltage to fix the angles of the galvanometer mirrors during the alignment.

12| Rotate the nosepiece to move an empty objective socket into the optical path for rough laser alignment. Adjust M_1 and M_2 to align the laser beam passing through the center of the objective socket. Place a glass cover slip on the microscope stage to reflect the laser beam back on its initial path. Use an infrared card with a hole in it to observe the reflected backward propagating laser beam for checking the laser incident angle. Install the Plan Apo ×60 objective in the optical path.
▲ CRITICAL STEP Use M_1 to adjust the laser center position and M_2 to adjust the laser incident angle.

13| Apply a small drop of immersion oil on the objective. Rotate the coarse-focus knob to lift the objective slowly to attach the immersion oil on the bottom surface of the glass cover slip until you observe the reflected laser on the monitor. Then rotate the fine-focus knob to lift the objective until you observe the laser focus spot hitting the top surface of the glass cover slip (observe the maximum intensity of the back reflection laser on the TV monitor).

14| Readjust M_1 to center the laser focus spot on the view field of the video CCD camera (CM_3), and then readjust M_2 to symmetrize the laser focus spot. Mark the laser position on the monitor and remove the glass cover slip.

15| Install the heating stage on the microscope and then place a test sample container with a glued quartz cover slip on it. Fill the sample container with distilled water and then add 5 µl of polystyrene spheres (2 µm in diameter, ~10^8 spheres per ml). Place a glass cover slip on the top of the sample container.

16| Repeat Step 13. Install the band-pass filter (F_2) in the optical path to block the reflected laser to the video camera. Turn on the LED. Rotate the fine-focus knob to lift the objective slowly to observe the phase-contrast images of the floating polystyrene spheres on the monitor.

17| Move the band-pass filter (F_2) out of the optical path to observe the reflected laser on the monitor. Rotate the fine-focus knob to lift the laser focus spot ~10 µm above the top surface of the quartz cover slip. Move the optical stage to obtain one floating sphere image in focus (sharp and bright image on the monitor) or near focus and near the laser beam. The strong laser gradient force should pull a nearby sphere into the laser focus and hold it.

18| Move the band-pass filter (F_2) into the optical path and then observe the monitor; the image of the trapped sphere should appear sharp, bright and stable. Smoothly move the microscope stage; the trapped sphere should not follow the movement. Lift or lower the objective, and the trapped sphere image should remain in the focus of the video CCD camera (CM_3). This will help to verify the optical trapping.

19| Move the position of lens L_3 slowly along the longitudinal axis and watch the image of the trapped sphere on the video monitor until the sphere's image is the sharpest and clear. This optimizes the optical tweezers.
? TROUBLESHOOTING

Perform confocal Raman spectroscopy of single trapped particles ● TIMING 10 h

20| *Align Raman scattering light on the entrance slit of the spectrograph (Steps 20–26):* Remove the heating stage and the test sample container from the microscope stage. Move the ×60 objective and the band-pass filter (F_2) out of the optical path. Turn off the LED. Install an ×10 objective into the optical path. Place and fix a near-infrared laser diode (850 nm) on the microscope stage above the objective and near the ×10 objective focus point.

21| Turn on the 850-nm laser diode. Move the microscope stage to center the 850-nm laser diode on the view field of the monitor. Rotate the focus knob to move the objective focus point to the 850-nm laser diode emitting surface. Readjust the microscope stage to overlap the 850-nm laser with the 780-nm trapping laser beam. The 850-nm laser should pass through the dichroic mirror (DM_1) for rough alignment of the Raman optical path.

22| Install the two lenses (L_5, f = 100 mm and L_6, f = 75 mm), with a distance of 175 mm, after the dichroic mirror (DM_1). Insert the pinhole (H, 100 μm) with an *XYZ* optics mount (combine the translation stage with the *XY* translator) between the two lenses as the spatial filter. Use the 850-nm laser to position the lens and the pinhole.

23| Install the 1D galvanometer mirror (GM_3). Power the galvanometer mirror's driver and apply 0 V control voltage to fix the angles of the galvanometer mirror during the alignment. Install the spectrograph (S). Adjust the galvanometer mirror (GM_3) to align the 850-nm laser passing through the central position of the entrance slit of the spectrograph (S).

24| Install the long-pass filter (F_4) in front of the spectrograph (S). Insert the focus lens (L_7, f = 75 mm) with an *XYZ* optics mount (combine the translation stage with the *XY* translator) between the 1D galvanometer mirror (GM_3) and the long-pass filter (F_4).

▲ **CRITICAL STEP** Carefully adjust the position of the focus lens (L_7) to ensure that the focus spot of the 850-nm laser is on the position of the entrance slit of the spectrograph (S).

25| Turn off the 850-nm laser diode and remove it from the microscope stage. Replace the ×10 objective with the ×60 objective in the optical path. Measure the laser power at the position between the *XY* galvanometer mirrors (GM_1, GM_2) and the scan lens, L_3. Rotate the half-wave plate (W_1) to adjust the laser power to 3 mW.

26| Open the WinSpec/32 software to start cooling the CCD camera on the spectrograph (S). Set the central wavelength of the spectrograph (S) to 900 nm. Calibrate the spectrograph according to the manufacturer's manual. Set the entrance slit of the spectrograph (S) to 50 μm.

27| *Optimize and calibrate Raman spectra of single trapped spheres (Steps 27–29):* Repeat Steps 15–18 to trap a polystyrene sphere.

28| Turn off the illumination light in the lab. Start to measure the Raman spectrum of the trapped polystyrene sphere. Adjust the two *XYZ* optical mounts of the pinhole (H) and the focus lens (L_7) to maximize the Raman signal.

▲ **CRITICAL STEP** Position the focus lens (L_7) to ensure that the Raman spectral image on the spectrograph's CCD chip is the brightest and the sharpest possible.

? TROUBLESHOOTING

29| Check the measured Raman spectrum of the trapped polystyrene sphere with the standard polystyrene Raman spectrum (strong Raman peaks at 620.9, 1,001.4 and 1,602.3 cm^{-1}) for fine calibration of the wave number of the spectrograph (S).

▲ **CRITICAL STEP** Although the spectrograph has been calibrated by the manufacturer, it is still necessary to check the accuracy of the calibration. Adjust the setting of the wavelength adjustment on the rear of the spectrograph to match the measured spectrum with the reference spectrum.

Measurement preparation ● TIMING 30–50 min

30| Replace the test sample container with a clean one. Add ~300 μl distilled water to the test sample container and then place a glass cover slip on the top of the sample container. Power the temperature controller to control the heating stage and then heat the distilled water inside the sample holder. Use the 10-KΩ thermistor to calibrate the temperature of the distilled water inside the sample container. Set the temperature controller to make the heating stage stable such that the distilled water in the sample container remains at 24 °C, the temperature we used for germination experiments, although higher temperatures could be used to speed up germination.

▲ **CRITICAL STEP** Calibrate the temperature controller every day before measurements.

31| Remove the glass cover slip. Add 3 µl of dormant *B. cereus* spores (~10^6 spores per ml) into the sample container. Replace the cover slip on the top of the sample container. The phase-contrast images of the floating spores should appear on the monitor.
▲ **CRITICAL STEP** Ensure that the added spores floating around the laser beam are as close as possible to the bottom of the quartz cover slip, and there are no air bubbles in the solution.

32| Move the band-pass filter (F_2) out of the optical path to observe the reflected laser on the monitor. Move the microscope to trap one spore. Move the band-pass filter (F_2) into the optical path, and the trapped dormant spore should appear circular and bright (**Fig. 2a**).

33| Install the cooled CCD camera (CM_1) on the left side port of the eyepiece tube mount to capture phase-contrast images. Set the full-view field of the recorded image of 1,600 × 1,200 pixels to the region of interest of 400 × 300 pixels, with the trapped spore's image in the center of the picture for fast display and acquisition. Keep this region of interest for the following measurements.

34| Move the emission filter (F_5) out of the optical path. Install the CCD camera (CM_2) to capture the bright-field image of the trapped spore. Zoom in the full-view field of the recorded image of 1,360 × 1,024 pixels to 340 × 256 pixels, with the trapped spore's image in the center of the picture for display. Keep this zoom in setup for subsequent fluorescence measurements. Place the emission filter (F_5) back into the optical path.

35| Acquire a Raman spectral image of the trapped spore with the full region 1,340 × 400 pixels of the spectrograph's CCD camera. Set the spectral region of interest of the spectrograph's CCD camera to 600 × 10 pixels to just cover the Raman spectral image region of the trapped spore.

Spore germination preparation ● TIMING 1 h
36| Take the test sample container out of the heating stage and place it in a clean and sterile sample container.
▲ **CRITICAL STEP** Owing to the high quality and cost of quartz microscope cover slips, the sample container with the quartz microscope cover slip is recycled. Fully clean and sterilize the sample container after each use.

37| Add 10 µl of dormant *B. cereus* spores (~10^6 spores per ml) to a 0.5-ml sterile flat-cap PCR tube. Place the tube in a digital-controlled water bath at 65 °C for 30 min to heat-activate the spores.

38| Place the flat-cap PCR tube containing the spores on ice for ≥ 15 min to cool the heat-activated spores.

39| Add 400 µl germinant solution of 1 mM L-alanine and 0.5 µM SYTO 16 in 25 mM Tris-HCl buffer (pH 7.4) to a 0.5-ml sterile flat-cap PCR tube. Place the tube in the digital-controlled water bath at 24 °C to preheat the germinant solution.

40| Add ~300 ml of 24 °C germinant solution to the sample container and add a new sterile glass cover slip on the top. Wait for 5 min for the system to reach thermal equilibrium.

41| Add 3 µl of heat-activated *B. cereus* (~10^6 spores per ml) spores to the germinant solution. Record the time and define this as time 0 of incubation.
▲ **CRITICAL STEP** The spore should be trapped as soon as possible; accordingly, observe the spores on the monitor soon after the spores are added.

Measurement of the germination of a trapped single spore ● TIMING 30 min to 2 h
42| Wait for 3 min for individual floating spores to diffuse into the germination solution. Move the band-pass filter (F_2) out of the optical path.

43| Trap individual spores with LTRS.
? TROUBLESHOOTING

44| *Single-trap LTRS*: Observe the laser focus position on the monitor and move the microscope stage to trap one individual spore (see the trapping process in Steps 17–18).

45| *Multi-trap LTRS*: Start the MATLAB program to control the multifunctional data acquisition device to generate the synchronous staircase waveforms and the trigger signal. Add the *m*-step and *n*-step staircase waveforms to scan the *XY* galvanometer mirrors (GM_1, GM_2) with a rate of 100 Hz. Observe the $m \times n$ array of laser traps on the monitor and move the microscope stage to trap $m \times n$ individual spores one by one. Add a synchronous ($m \times n$)-step staircase waveform to steer the GM_3 and add the trigger signal to the spectrograph (S).

46| Place the band-pass filter (F_2) back into the optical path. Start measurements of the Raman spectra, phase-contrast images and fluorescence images of the trapped spore to follow the germination of the trapped spore.
? TROUBLESHOOTING

47| Use three computers for the real-time display and storage of recorded Raman spectra, phase-contrast images and fluorescence images of the trapped spore, respectively.

48| Use the Visual Basic program (phase-contrast image CCD control program (see **Box 1** and the **Supplementary Methods** file) to extract a small portion (21 × 21 pixels) of the recorded phase-contrast images of the trapped spore at each time of measurement (**Fig. 5b**). Average the image intensities of these 441 pixels and then subtract the nearby averaged background values to get the phase-contrast intensities. Plot the curve of phase-contrast intensity versus time for a real-time display of the refractility change (**Fig. 5d**).

49| Use the MATLAB program to extract a small portion (25 × 25 pixels) of the recorded fluorescence images of trapped spores (**Fig. 5c**), average the values of all these spore-centered 625 pixels at each time of measurement and then subtract the nearby averaged background values to get the fluorescence intensity values. Plot the curve of fluorescence intensity versus time for a real-time display of the fluorescence change (**Fig. 5d**).

50| After you finish recording the spore's germination, release the trapped spore. Record 20 frames of background Raman spectra. Average these 20 frames to get the smoothed background Raman spectrum.

51| Use the MATLAB program to subtract the background Raman spectrum from the recorded time-lapse Raman spectra. Then, add five values of Raman intensities of the band at 1,017 cm^{-1} (centered value at 1,017 cm^{-1} and the adjacent four values, after the subtraction of the smoothed intensity at 990 cm^{-1}) due almost completely to CaDPA for each Raman spectrum to calculate the CaDPA level in the trapped spore. Plot the curve of CaDPA level versus incubation time for the entire spore germination process.

Use of phase-contrast microscopy to measure $T_{release}$ of large numbers of germinated spores ● TIMING 1–3 h
52| Turn off the laser. Remove the sample container from the heating stage.

53| Take a clean, sterile sample container with the bottom glued to a glass cover slip (a glass cover slip is used here as no Raman measurements are taken in this step). Place 10 μl of heat-activated *B. cereus* spores (~3×10^9 spores per ml) on the glass cover slip. Place the sample container in a refrigerator at 4 °C for 5 min to allow the spores to adhere.

54| Use a micropipette to remove the spore suspension and leave a thin film of spores on the surface of the cover slip. Place the sample container in a vacuum desiccator at < 0.01 atm for 5–15 min to dry the thin film of spores.

55| Place the sample container on the heating stage of the microscope. Wait for 5 min for the cover slip temperature to stabilize at 24 °C.

56| Add germinant solutions with 10 mM or 0.1 mM L-alanine in 25 mM Tris-HCl buffer (pH 7.4) at 24 °C to the sample container. Record the time and define this as time 0 of incubation. Place a glass cover slip on the top of the sample container.

57| Record the full-view field (1,600 × 1,200 pixels; an area of 192 μm × 144 μm) of the phase-contrast image at a rate of 1 frame per 15 s, with an exposure time of 0.2 s, to follow the germination of large numbers of individual *B. cereus* spores adhered on the cover slip.

58| After the measurement, analyze the recorded text images.

59| Use the MATLAB program to locate and label each dormant spore with the first recorded text image. The dormant spores will appear in a brighter phase compared with the environment. Find the border of each dormant spore (blue circle curves, as

shown in **Fig. 6a**). The sum of the pixel values inside each border is the phase-contrast intensity of the corresponding spore. Calculate time-lapse phase-contrast intensities of each spore from the recorded time-lapse phase-contrast images. Import these calculated data.

60| Use Origin software to plot the curves of phase-contrast intensity versus the incubation time for the refractility changes of the individual spores during germination. Position the $T_{release}$ time for each spore manually from the spore's refractility change curve as the end of the rapid drop in refractility (**Fig. 6c**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
19	Weak trapping	Scan lens L_3 is not in the correct position	Carefully move the scan lens along the axial direction back and forth to find the best position for strong trapping. Make sure that the laser position on the monitor is not changed during changes of position of the scan lens
28	High Raman background	Background light from the monitor or interference from the scattering laser	Build one large box with black fiberboard to cover the whole laser and the Raman optical path to block background light from the monitor. Build another small box with black fiberboard (inside the larger box) to cover the optical path of the focus lens L_7, filter F_4 and the spectrograph to prevent interference from the scattering laser
43,46	Cannot find spores or other surrounding floating spores enter the laser trap and collide with the trapped spore	Too few or too many added spores. With too few added spores, it takes a long time to find a spore near the laser focus spot for trapping in Step 43. However, too many spores greatly increases the chance of the floating spores colliding with the trapped spores	The concentration and volume of added spores must be controlled. In our experience, when spores are added in 3-μl volumes, the optimal spore concentration is ~10^6 spores per ml. In Step 43, after a single spore is trapped, move the trapped spore out of the center of concentration of the added spores. In Step 46, watch the monitor to move the trapped spore away from nearby floating spores. Normally, 10–20 min after spore addition, most of the spores sink to the bottom of the germination solution and some become attached to the surface of the quartz cover slip

● TIMING

Steps 1–19, Construct optical tweezers with the external phase-contrast microscope: 16 h
Steps 20–29, Perform confocal Raman spectroscopy of single trapped particles: 10 h
Steps 30–35, Measurement preparation: 30–50 min
Steps 36–41, Spore germination preparation: 1 h
Steps 42–51, Measure the germination of a trapped single spore: 30 min to 2 h
Steps 52–60, Simultaneous measurement, using phase-contrast microscopy, of the time ($T_{release}$) for the completion of CaDPA release from large numbers of germinated spores: 1–3 h

ANTICIPATED RESULTS

CaDPA level and phase-contrast intensity of dormant spores

For dormant *B. cereus* spores, Raman spectral peaks at 662, 824, 1,017, 1,395 and 1,572 cm^{-1} (**Figs. 2b**, **3b** and **5a**) are obtained primarily because of the presence of CaDPA[30]. The intensities of these bands are directly proportional to a spore's CaDPA level, and spores with higher CaDPA levels have higher phase-contrast image intensities. A direct correlation between the CaDPA level and the phase-contrast intensity of single trapped dormant *B. cereus* spores has been demonstrated with the combined system of phase-contrast microscopy, Raman microscopy and optical tweezers[34].

CaDPA release, loss of refractility and change of fluorescence during spore germination

The time-lapse Raman spectra, phase-contrast images and fluorescence images during germination of a single trapped *B. cereus* spore can be recorded simultaneously, as shown in **Figure 5a–c**. The overall germination process is then followed (**Fig. 5d**) by simultaneously measuring: (i) the release of CaDPA calculated from the intensity of the CaDPA-dominated Raman

scattering band at 1,017 cm^{-1}; (ii) the loss of refractility calculated from the intensities of phase-contrast images; and (iii) the binding of SYTO 16 to spore DNA measured by fluorescence intensities. The correlations between these three different measurements reveal that: (i) the end of stage I of germination, determined by end of the rapid drop in spore refractility, corresponds to the completion of CaDPA release[34,] and (ii) SYTO 16 uptake is minimal until CaDPA has been released, but rapid SYTO 16 uptake and binding to spore DNA begins during hydrolysis of the spore's peptidoglycan cortex[37].

Germination heterogeneity

$T_{release}$, the time for complete release of a spore's CaDPA, is a characteristic time in spore germination and can be used to express the germination heterogeneity of the spores[32,34]. As noted above, the $T_{release}$ time can be obtained by phase-contrast microscopy. In addition, it is easy to get $T_{release}$ values for large numbers of individual germinating *B. cereus* spores from phase-contrast images of spores germinating when adhered on a glass cover slip (**Fig. 6a**,**b**) instead of using Raman spectroscopy. **Figure 6c** shows examples of germination curves of seven *B. cereus* spores determined by the loss of refractility through their phase-contrast images, and values of $T_{release}$ are correctly positioned, as indicated by the vertical red arrows. The distribution of $T_{release}$ values for 500 individual spores germinating with 10 mM L-alanine (red) and for 900 individual spores germinating with 0.1 mM L-alanine (blue) are shown in **Figure 6d**. This analysis reveals that, with the lower germinant concentration, spore germination exhibits a much broader distribution of $T_{release}$ time periods compared with the higher germinant concentration[34,41].

ACKNOWLEDGMENTS This work was supported by a grant from the Army Research Office (Y.-q.L. and P.S.) and by a Multidisciplinary University Research Initiative award from the United States Department of Defense (P.S. and Y.-q.L.).

AUTHOR CONTRIBUTIONS L.K. and Y.-q.L. designed the experimental setup, performed the research, analyzed data and wrote the manuscript. P.Z., G.W. and J.Y. performed the experiments and analyzed data. P.S. provided spore samples, supervised the microbiological work and revised the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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